REMARKS

In the Office Action dated October 16, 2008, claims 1-32 are pending and under consideration. Claims 1-12, 14, and 16-32 are rejected. Claims 13 and 15 are objected to.

This Response addresses each of the Examiner's rejections and objections. Applicant therefore respectfully submits that the present application is in condition for allowance. Favorable consideration of all pending claims is therefore respectfully requested.

Claim Objections Under 37 C.F.R. §1.75(c)

Claims 13 and 15 are objected to under 37 C.F.R §1.75(c) as improper multiple dependent claims, because these claims depend from other multiple dependent claims.

In response to the objection, claim 13 has been canceled and claim 15 has been amended. Withdrawal of the objection is therefore respectfully requested.

Claim 24 is objected to under 37 C.F.R §1.75(c) as improper dependent claim for failing to further limit the subject matter of the claim from which claim 24 depends. Additionally, claim 24 does not depend from a preceding claim.

Claim 24 has been canceled without prejudice. Withdrawal of the objection thereof is respectfully requested.

Rejection Under 35 U.S.C. §101

Claims 31-32 are rejected under 35 U.S.C. §101 as improper process claims without setting forth any steps.

Claims 31-32 have been canceled without prejudice. Withdrawal of the rejection is therefore respectfully requested.

Rejections Under 35 U.S.C. §112, Second Paragraph

Claims 31-32 are rejected under 35 U.S.C. §112, second paragraph, as being indefinite. The rejection is moot in view of the cancellation of these claims.

Claim 25 is rejected under 35 U.S.C. §112, second paragraph, because the term "said subject" is alleged to lack antecedent basis. Applicant submits that the amendment to claim 25 has overcome this rejection.

Claim 16 is rejected under 35 U.S.C. §112, second paragraph, because it is allegedly unclear as to how the method step could result in the modulation of a lymphocyte.

In response, Applicant has deleted the term "lymphocyte" from the preamble of claim 16 and has replaced the term "said cell" at the end of the claim with "said APC". Applicant submits that the amendment to claim 16 has overcome this rejection. It should be noted, however, that APC lysis or death ultimately could affect the function of lymphocytes.

Claims 27-30 are rejected under 35 U.S.C. §112, second paragraph, as it is allegedly unclear how contacting a graft with the recited agent, as claimed in claims 27-30, would result in the therapeutic treatment of a condition without, for example, specifying that the contact occurs *in vivo* in the subject.

Applicant submits that the specification discloses contacting APCs both *in vivo* and *in vitro* (see, for example, page 22, line 28 to page 23, line 8 of the specification). Accordingly, currently amended claim 27 is directed to specifying that the contact occurs *in vivo*, and newly added claim 33 is directed to specifying that the graft is contacted *in vitro*, prior to implantation.

In view of the foregoing, it is respectfully submitted that the rejections under 35 U.S.C. § 112, second paragraph are overcome, and withdrawal thereof is respectfully requested.

Rejection Under 35 U.S.C. §112, First Paragraph—Written Description

Claims 1-12, 14 and 16-30 are rejected under 35 U.S.C. §112, first paragraph, as failing to comply with the written description requirement.

Independent claims 1 and 20 are drawn to a method of employing an "agent" that binds to a cell-surface molecule. Certain dependent claims refer to this "agent" or alternatively to an "immunointeractive molecule". The Examiner contends that these terms are too broad because the specification only discloses antibodies.

In response, claims 1 and 20 have been amended to delete the term "agent" and to recite specifically "an antibody or antigen-binding fragment thereof". Claims 6-7, 13 and 23 have been canceled, and claims 8, 10 and 15 have been amended. Additionally, the term "monoclonal antibody" in claim 16 and the antibodies referred to in claims 25-27 have been amended to reference antigen-binding fragments of antibodies.

Claims 1-8, 11-12, 16 and 18-23 are also drawn to methods comprising contacting an APC with an antibody that binds to a "cell-surface activation molecule", a "cell surface antigen" or an "APC surface activation molecule". The Examiner has objected to these terms as overly broad. According to the Examiner, the specification only discloses a single species of cell surface activation molecule, CMRF-44, a marker of dendritic cells.

In response, claims 1, 16 and 20 have been amended to specify that the molecule/antigen is CMF-44. In addition, claims 9, 17 and 24 have been canceled without prejudice, and dependent claims 10 and 18 have been amended accordingly.

The Examiner has also objected to the term "functional equivalents" of an antibody recited in claims 7-10 and 14, because the Examiner contends that only antigen-binding antibody

fragments are adequately disclosed in the specification. Applicant submits that the amendments to the claims presented herein have obviated this rejection.

The Examiner has also objected to the term "functional equivalents" of CMRF-44 recited in claims 17-19 and 24-30, allegedly because the specification does not disclose such equivalent of CMRF-44. Applicant has amended the claims to delete this term.

In view of foregoing amendments, the written description rejection under 35 U.S.C. §112, first paragraph, is overcome and withdrawal thereof is respectfully requested.

Rejection Under 35 U.S.C. §112, First Paragraph—Enablement

Claims 1-12, 14, 16-30 are rejected under 35 U.S.C. § 112, first paragraph, for allegedly lacking enablement.

The Examiner has rejected the claims because the specification allegedly does not provide enablement for the claimed methods insofar as the terms "agent", "immunointeractive molecule", "cell-surface activation molecule", "cell surface antigen", "APC surface activation molecule", "functional equivalent" of an antibody and "functional equivalents" of CMRF-44 are concerned.

Applicant believes that the amendments presented herein to overcome the written description rejection, as discussed above, have also overcome the lack of enablement rejection as well insofar as the above terms are concerned.

The Examiner has also rejected claims 1-12, 14 and 16-23, because the Examiner considers the term "modulating" to encompass "up-regulation" as well as "down-regulating", and thus the Examiner is of the opinion that the claimed methods of "modulating" are not enabled.

In response, the term "modulating" in claims 1, 16 and 20 have been replaced with "preventing, inhibiting or otherwise down regulating" to overcome the rejection.

Additionally, the Examiner rejects claims 1-8, 11-12, 16 and 18-19 as the claims do not indicate the "cell-surface activation molecule" or "cell surface antigen" are those expressed by APCs. Applicant submits that the amendments specifying CMRF-44 in claim 1, 16 and 20 are believed to have overcome this rejection.

Furthermore, the Examiner also contends that claims 1 and 2 encompass modulating APC activity by methods other than inducing cell lysis. Applicant respectfully submits that cell lysis is believed to be a principal mechanism underlying the observed down-regulation; however, there is no need to recite the underlying mechanism.

Finally, the Examiner has also questioned as to how the method of claim 16, as performed, could result in the modulation of a lymphocyte. As discussed in the context of the rejection under 35 U.S.C. § 112, second paragraph, the term "lymphocyte" has been deleted from the preamble of claim 16.

In view of the foregoing, it is respectfully submitted that the enablement rejection under 35 U.S.C. § 112, first paragraph is overcome and withdrawal thereof is respectfully requested.

Rejections Under 35 U.S.C. §102(b)

Koppi

Claims 1-12 and 16-19 are rejected under 35 U.S.C. §102(b) as anticipated by Koppi et al., 2001, *Immunol. and Cell Biol.*. The Examiner alleges that Koppi et al. teach a method of inducing dendritic cell lysis (i.e., down-regulating the immuno-activity of an APC) comprising contacting human dendritic cells with a monoclonal antibody specific for CMRF-44.

Applicant respectfully submits that Koppi et al. disclose that the PBMC cells were labelled with the CMRF-44 antibody and incubated with complement, resulting in cell death.

Therefore, the reference teaches that death of dendritic cells ("DC") is caused by the complement and not by the CMRF-44 antibody. In addition, Koppi et al. comment that "CMRF-44 [antibody] may be a suitable candidate for a novel immuno-suppressive strategy, targeting DC instead of lymphocytes" at the end of abstract of disclosure. Applicant submits that this comment is apparently made based on the discovery that CMRF-44 identified a unique subgroup of DCs, independent of lymphocytes, as the disclosure does not show that the CMRF-44 antibody *by itself* had an immunomodulatory effect on DCs.

Therefore, Applicant submits that Koppi et al. do not teach down-regulating of an APC by employing a CMRF-44 antibody, as presently claimed. The reference simply demonstrates that the CMRF-44 antigen is specific to a particular DC subgroup. Withdrawal of the rejection of Claims 1-12 and 16-19 under 35 U.S.C. §102(b) based on Koppi et al. is respectfully requested.

WO 99/24078

Claims 1-8, 11-12 and 16-30 are rejected under 35 U.S.C. §102(b) as anticipated by WO 99/24078. The Examiner alleges that WO 99/24078 teaches a method of down-regulating the immuno-activity of an APC by contacting the APC with an antibody specific for an antigen expressed by APCs. The reference also teaches antibodies specific for CD11c, which the Examiner alleges to be a "functional equivalent" of CMRF-44 since both CMRF-44 and CD11c display a similar expression pattern (i.e. they are DC-specific makers).

Applicant respectfully submits that the claims have been amended to be drawn specifically to antibodies (and antigen-binding fragments thereof) specific for CMRF-44, and has deleted the term "functional equivalents" of CMRF-44. The Examiner has acknowledged in the Office Action that the reference does not disclose an antibody specific for CMRF-44.

Accordingly, WO 99/24078 does not teach the methods, as presently amended. Withdrawal of the rejection under 35 U.S.C. §102(b) based on WO 99/24078 is respectfully requested.

Rejection Under 35 U.S.C. §103(a)

Claims 1-12, 14 and 16-30 are rejected under 35 U.S.C. §103(a) as allegedly obvious over WO 99/24078 in view of U.S. Patent No. 5,876,917, as evidenced by Flavell et al., *Cancer Research*, 58, 5787-5794, December 15, 1998.

The Examiner alleges that WO 99/24078 teaches a method of down-regulating the immuno-activity of an APC by contacting the APC with an antibody specific for an antigen expressed by APCs, but admits that this application does not teach an antibody specific for CMRF-44. The Examiner apparently has attempted to rely on the '917 patent to cure the deficiencies of WO 99/24078. The '917 patent teaches an antibody specific for CMRF-44 which is expressed by activated APCs. The '917 patent further teaches that CMRF-44 is a marker that can be used to specifically identify all stimulatory populations of APCs.

Applicant respectfully submits that the claimed subject matter is not obvious over the cited references taken in combination. The mere knowledge of the existence of an APC-binding antibody would give the skilled artisan ***no*** expectation that such antibody would be useful for modulating the function of cells that the antibody bound to.

For example, Applicant refers the Examiner to Hock et al. (1999) *Tissue Antigens*, 53: 320-334 (attached hereto as **Exhibit 1**), which describes the DC antigen CMRF-56. Hock et al. demonstrated that human dendritic cells express a 95 kDa activation/differentiation antigen that is defined by CMRF-56. However, CMRF-56 antibodies do not immunomodulate their DCs, which is an essential feature that permits the use of CMRF-56 antibodies to immnuo-select a

subpopulation of DCs for use in immunotherapy. See, for example, Freeman et al. (2007) *Journal of Immunology*, 30 (7): 740-748 (attached hereto as **Exhibit 2**).

In view of the foregoing, the rejection of claims 1-12, 14 and 16-30 under 35 U.S.C. §103(a) as allegedly obvious over WO 99/24078 in view of U.S. Patent No. 5,876,917, is overcome. Withdrawal of the rejection is therefore respectfully requested.

Non-Statutory Double Patenting Rejection

Claims 1-12, 14, and 16-30 are provisionally rejected on the ground of non-statutory obviousness-type double patenting as unpatentable over claims 1-6, 8-13, 15, and 20 of copending Application No. 10/524,716, in view of U.S. Patent No. 5,876,917.

Applicant acknowledges that the rejection is provisional in nature and can be overcome by timely filing a terminal disclaimer.

Conclusion

In view of the foregoing amendments and remarks, it is firmly believed that the subject application is in condition for allowance, which action is earnestly solicited.

Respectfully submitted,

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EXHIBIT 1

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Human dendritic cells express a 95 kDa activation/differentiation antigen defined by CMRF-56

Key words:
antigen; CMRF-56; dendritic cell; monoclonal antibody

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Abstract: Despite the unique functions of dendritic cells (DC), only two cell surface antigens (CMRF-44 and CD83) with relatively restricted expression on human DC have been described to date. We describe a third mAb, CMRF-56, which recognizes another DC early activation/differentiation antigen with limited expression on other haemopoietic cell populations. Circulating blood leukocytes did not express the CMRF-56 antigen and, following either *in vitro* culture or activation of PBMC populations, CMRF-56 antigen expression was detected only on DC and a subpopulation of $CD19^+$ lymphocytes. Circulating blood DC were $CMRF\text{-}56^-$ but induced expression within 6 h of *in vitro* culture. This, together with the finding that tonsil and synovial fluid DC upregulate the antigen following short-term *in vitro* culture, confirmed that CMRF-56 recognizes an early activation antigen on DC. Isolated Langerhan's cells, dermal DC, migratory dermal DC and monocyte derived DC (GM-CSF/IL-4/TNFα) also express the CMRF-56 antigen. Antigen modulation studies demonstrated that the amount of cell surface bound CMRF-56 and CMRF-44 (but not CD83) mAb was dramatically reduced by short-term incubation at 37°C. This effect was not due to internalization and the reduction in CMRF-56 binding was a reversible, temperature-dependent process. In contrast, the decrease in CMRF-44 binding was irreversible, suggesting that following ligation the CMRF-44 antigen undergoes an irreversible conformational change or shedding at 37°C. Western blotting confirmed that CMRF-56 recognizes a previously undescribed 95 kDa activation antigen whose cellular distribution and expression kinetics overlaps with, but is clearly distinguishable from, that of the CD83 and CMRF-44 antigens. CMRF-56 therefore provides a useful additional marker for studies on human DC.

Dendritic cells (DC) are potent antigen-presenting cells (APC) whose central importance in the initiation of immune responses is now well established (1, 2). These cells are present in low numbers in both lymphoid and non lymphoid tissue and constitute <1% of peripheral blood leukocytes. Although it is clear that the different tissue DC populations and their precursors are derived from bone marrow (BM) stem cells (3, 4) the normal growth and differentiation pathway of DC *in vivo* is unknown. It is unclear whether the different DC populations within tissue represent different maturation

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stages of a DC lineage or whether they stem from distinct progenitors. Following *in vitro* culture in cytokines, DC-like populations can be generated from various haemopoietic cell populations including human bone marrow, cord blood or mobilized progenitors (5–7) and similar monocyte derived DC populations (Mo-DC) can also be generated from both adherent peripheral blood mononuclear cells (PBMC) and purified monocyte populations (8–11). The relationship of these *in vitro* generated DC to other *in vitro* and *in vivo* DC populations remains unclear.

DC populations are defined functionally by their ability to stimulate T-lymphocyte responses and phenotypically by their high density expression of adhesion molecules and MHC class II gene products, in conjunction with a lack of defined cell lineage markers notably CD3, CD14, CD16 and CD19 (hereafter lin$^-$) (1). Studies on directly isolated blood DC have shown that these preparations contain subsets, differing both in their functional capacities and their expression of antigens such as CD33, CD4, CD11c, CDw123 and CMRF-44 (12–15). This is thought to reflect differences in the differentiation/activation state of these cells and *in vitro* activation has been shown to induce rapid changes in the morphology, phenotype and function of both blood DC (12–16) and LC (17, 18). Similar maturation is thought to occur *in vivo* as DC acquire antigen and migrate to the T-lymphocyte areas of lymphoid tissue.

The activation of DC populations rapidly induces the expression of the cell surface antigens CMRF-44 and CD83. These antigens have been shown to provide relatively selective markers of activated DC populations (15, 19–21) and their expression has also been demonstrated on *in vitro* cytokine generated Mo-DC (9–11). The relative kinetics of CMRF-44 and CD83 antigen expression and their expression at different stages of DC differentiation has not been established as yet. Delineation of the stages of DC differentiation/activation is critical to improved understanding of DC ontogeny and function. This is the first report describing a new DC differentiation/activation antigen, defined by the monoclonal antibody CMRF-56, which has limited expression on other haemopoietic populations. The kinetics of its expression on DC has been compared to that of the CD83 and CMRF 44 antigens during DC differentiation/activation.

Material and methods

Monoclonal antibodies and immunolabelling

The monoclonal antibodies CMRF-2 (CD71, IgG_1), CMRF-15 (erythrocyte sialoglycoprotein, IgM), CMRF-31 (CD14, IgG2a), CMRF-11 (CD45 RA, IgG1), CMRF-26 (CD45, IgG1), CMRF-44 (IgM) and biotinylated CMRF-44 were produced in our laboratory. HB15a (CD83, IgG2b), both unconjugated and phycoerythrin (PE) conjugated were obtained from Immunotech (Marseilles, France). The CD19 mAb FMC63 (IgG2a) and the isotype control mAb X63 (IgG1), Sal4 (IgG2b) and Sal5 (IgG2a) were a gift from Prof. H. Zola (Flinders Medical Centre, Adelaide, Australia). The CD1a mAb Na1/34 was a gift from Prof. A. McMichael (Institute of Molecular Medicine, Oxford, UK). HuNK-2 (CD16, IgG2a) was a gift from Prof. I. McKenzie (Austin Research Institute, Melbourne, Australia). OKT3 (CD3, IgG2a), HNK-1 (CD57, IgM), G28–5 (CD40, IgG1) and OKM1 (CD11b, IgG1) were produced from hybridomas obtained from the ATCC. PE-conjugated antibodies to CD3 (leu4, IgG1), CD14 (leuM3, IgG2b), CD16 (leu11c, IgG1), CD19 (leuM12, IgG1) and HLA-DR (L243, IgG2a) antigens were purchased from Becton Dickinson (San Jose, CA, USA). The mAb CMRF-44, CMRF-56 and G28–5 (CD40) were fluorescein isothiocyanate (FITC) conjugated by incubation (2 h, RT) of 1 ml mAb (2 mg/ml in 0.1 M Boric acid, pH 9.6) with 30 µl FITC isomer I (10 mg/ml in dimethyl sulfoxide (DMSO)) followed by dialysis. FITC and phycoerythrin (PE) conjugated sheep anti-mouse immunoglobulin (FITC-SAM, PE-SAM) were purchased from Silenus (Hawthorn, Australia). FITC-conjugated CD71 specific mAb were obtained from Becton Dickinson and FITC-conjugated CD86 mAb from PharMingen (San Diego, CA, USA). FITC-conjugated rabbit anti-sheep (RAS) was obtained from Dako (Carpenteria, CA, USA). Labelling was carried out on ice as described previously (19, 22).

Generation of the CMRF-56 mAb

A balb/c mouse was immunized with the HD-derived cell line L428 and the splenocytes fused with the myeloma line NS-1 four days later. The CMRF-56 hybridoma was cloned three times by limiting dilution and used to generate ascites fluid. Isotyping was performed using an indirect ELISA kit (Sigma, St. Louis, MO, USA). CMRF-56 was purified using a Ifi Trap Protein A column (Sigma) and biotinylated using Biotin-X-NHS (Calbiochem, La Jolla, CA, USA). Briefly CMRF-56 at 2 mg/ml in 0.05 M $NaHCO_3$ (pH 8.5) was incubated with Biotin-X-NHS (7.5 µg/ml, Calbiochem) for 30 min (RT) prior to dialysis.

Cell lines

T-cell lines (HSB-2, Molt 4 and Jurkat), Epstein-Barr virus (EBV)-transformed B-cell lines (WT49, Mann), Burkitt's lymphoma lines (Raji and Daudi), pre-B (Nalm 6), myeloerythroid (K562) and monocytoid leukemia (HL60, U937, KG1, KG1a, THP-1, HEL) cell lines were grown in medium (10% fetal calf serum (FCS); Irvine Scientific, Santa Anna, CA, USA) in RPMI-1640 (Gibco, Auckland, New

Zealand) supplemented with 2 mM glutamine, 60 mg/l penicillin and 0.1 g/l streptomycin). The Hodgkins cell line L428 was obtained from Dr. V. Diehl (Clinik for Innere Medizine, Cologne, Germany) and the Hodgkins cell lines KM-H2 and HDLM-2 (grown in 20% FCS) were obtained from Dr. H. G. Drexler (German Collection of Micro-organisms and Cell Cultures, Braunschweig, Germany).

Lymphocyte, granulocyte and monocyte preparation

Blood was obtained from volunteer donors with appropriate informed consent according to Ethical Committee guidelines. Peripheral blood mononuclear cells (PBMC) and granulocytes were prepared as described previously (22). T-lymphocyte-enriched fractions (ER^+) and non-T-cell fractions (ER^-) were prepared from PBMC by rosetting with neuraminidase treated sheep erythrocytes as described previously (22). Granulocytes were activated by culture (15 min, 37°C) at 2×10^6/ml in medium (10% FCS/RPMI) supplemented with 1 μM N-Formyl-Met-Leu-Phe (FMLP) (Sigma). Activated T lymphocytes were prepared by culture of: (A) ER^+ PBMC (2×10^6/ml) in medium supplemented with either 5 μg/ml PHA (Sigma) or the phorbol ester phorbol 12 – myristate 13 acetate (PMA) (Sigma) at 25 ng/ml plus the calcium ionophore A23187 (Sigma) at 500 ng/ml; or (B) PBMC at 2×10^6/ml in medium or medium plus IL-2 (20 U/ml; Hoffman-La Roche, Basel, Switzerland) for 72 h in round bottomed plates (96-well, Nunc) precoated (1 h, 37°C) with OKT3 (CD3) at 1 μg/ml. T-lymphocyte populations were monitored by double labelling with CD3-PE.

ER^- PBMC were used as an enriched source of monocytes. Activated monocytes were obtained by culture of ER^- PBMC (2×10^6/ml) in medium supplemented with either IFNγ (500 U/ml; a gift from Boehringer Ingelheim, Germany), bacterial LPS (100 ng/ml; Sigma), TNFα (20 ng/ml; Hoffman La Roche) or rhGM-CSF (500 μ/ml; Sandoz-Pharma, Auckland, New Zealand). Monocyte populations were monitored by double labelling with CD14-PE.

The effectiveness of the *in vitro* activation was determined by monitoring changes in the expression of the activation antigens CD25, CD71, HLA-DR and CMRF-44 by flow cytometry. Granulocyte activation was assessed by monitoring changes in CD11b expression.

Dendritic cell preparation

Highly enriched DC populations were prepared using established laboratory methods:

i) *Fresh blood DC*

Resting DC were prepared by direct immunodepletion (15, 23). Briefly, ER^- PBMC were labelled with a mix of CD3, CD11b, CD14, CD16 and CD19 mAb. After incubation with MACS magnetic microspheres (Miltenyi Biotech, Bergisch Gladbach, Germany), labelled cells were removed by magnetic immunodepletion and the mAb-negative cells (lin^-) were then labelled with FITC-SAM and further purified by FACS sorting. In a number of experiments resting DC were then cultured (37°C, 5% CO_2) at 2×10^6/ml in medium (10% FCS/RPMI) prior to analysis.

ii) *Cultured blood DC*

Cultured low density blood DC were prepared from cultured (16 h, 37°C, 5% CO_2) ER^-PBMC (15). The low density fraction was then isolated by centrifugation over a Nycodenz (Nycomed Pharma, Norway) gradient and used either directly as a DC enriched (10–30%) fraction or further purified by immunodepletion as described above.

iii) *Skin DC*

Langerhan's cells (LC) and dermal DC were isolated as previously described (24) from skin (obtained with appropriate ethical permission as approved by the Canterbury Health Ethical Committee) separated into epidermal sheets and dermis by overnight digestion (4°C) with dispase (0.25% in PBS, Boehringer-Mannheim, Mannheim, Germany). Epidermal cell suspensions were produced by incubation of epidermal sheets in 0.25% Trypsin (Sigma) at 37°C for 20 min followed by vigorous pipetting and disaggregation through a cell dissociation cup (grade 40 mesh; Sigma). Fresh LC were enriched (10–40%) at this stage by a lymphoprep gradient (25). Fresh dermal DC were prepared from the cell suspensions obtained from minced dermal sheets by incubation (1 h, 37°C), with collagenase D (1 mg/ml; Boehringer-Mannheim) and DNAase I in medium. A single cell suspension was obtained by filtering through nylon mesh (80 μm) and following centrifugation over a lymphoprep gradient ($d=1.077 g/cm^3$, 10 min, 500×*g*) the low density fraction was utilized as an enriched (30–50%) dermal DC population. Migratory dermal DC were isolated from cultured dermis. Briefly, dermis was fragmented and cultured (37°C, 5% CO_2) in medium for 48 h. Following removal of dermal fragments by filtration through nylon gauze, DC were enriched (80–90%) by centrifugation over a Nycodenz gradient. The DC populations within these enriched preparations were identified by their expression of MHC class II.

iv) *Synovial fluid DC*

Synovial fluid DC (SFDC) were isolated as previously described (26). Following informed consent SF was collected by routine knee joint aspirations from patients with chronic arthritis. ER^- cells were labelled with a mix of mAb against CD3, CD14, CD15, CD16 and CD19 and depleted using immunomagnetic MACS beads and FACS sorting as described above for preparation of blood DC. The remaining unlabelled MHC class II positive cells constituted the SFDC population.

v) *Tonsil DC*

Tonsil DC were prepared from tonsils obtained at routine tonsillectomies, following informed consent. These were processed immedi-

ately and a single cell suspension prepared by mincing the tissue finely and passing the material through a wire mesh sieve. Mononuclear cells were isolated over a F/H density gradient and tonsil DC isolated as described above for SFDC.

vi) *Monocyte-derived DC (Mo-DC)*

Mo-DC were generated from the adherent fraction of PBMC or ER^- PBMC obtained following a 2 h culture (37°C) in Falcon 6-well plates (BD). Adherent cells were cultured in medium supplemented with rhGM-CSF (800 U/ml; Sandoz-Pharma) and rhIL-4 (500 U/ml; Sigma) for five days, followed by addition of either TNFα (20 ng/ml; Hoffman-La Roche) or LPS (1 μg/ml; Sigma) and culture for a further two days.

Immunohistology

Indirect immunoperoxidase staining of cryostat cut sections (7 μm) of tonsil and lymph node (obtained with consent) was performed as reported previously (27). Immunofluorescent double labelling of acetone fixed tissue sections was carried out as described above for cell suspensions.

Preparation of CD83 transfectants

COS-7 cells were transfected by electroporation (300 V, 500 μF) of cells (4×10^6 in 400 μl medium) with 2 μg of CD83 plasmid (CDM8 – CD83 kindly provided by Dr. T. F. Tedder, Duke University Medical Center, Durham, NC, USA) in a Biorad Gene Pulser. Cells were then cultured in medium 72 h prior to immunofluorescent analysis with the HB15a mAb to confirm CD83 expression.

Functional assays

Allogeneic mixed leukocyte reaction (MLR): 10^5 T lymphocytes were cultured at 37°C in 5% CO_2 in 96-well plates with triplicate graduated numbers of sorted APC subsets obtained from a single allogeneic donor. Wells were pulsed for 12 h with 0.5 μ Ci tritiated thymidine (Amersham) immediately prior to harvest at five days. Cells were harvested onto glass fibre paper and thymidine incorporation was measured with a liquid scintillation counter. Data are expressed as mean CPM of triplicate wells ±SD. Control wells containing T cells or APC alone incorporated <500 cpm of tritiated thymidine in all experiments.

Immunoprecipitation and Western blotting

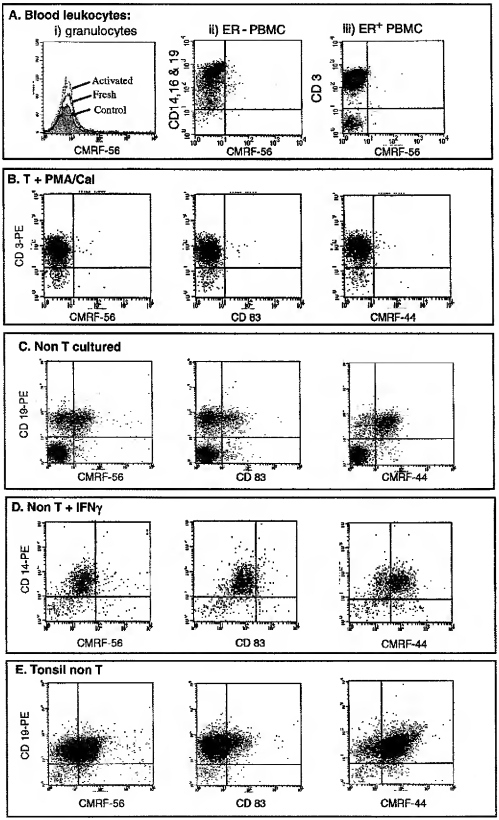
For immunoprecipitation studies, cells were labelled by either (i) cell surface labelling with Biotin-X-NHS (Calbiochem) (19), (ii) cell surface sialic acid labelling with biotin hydrazide (Calbiochem) (28) or (iii) biosynthetically labelled with ^{35}S (NEN, Boston, MA, USA) (19). Following labelling cells were solubilised by incubation (1 h on ice) of cells (4×10^7) in 1 ml lysis buffer (100 mM Tris, 150 mM NaCl, 0.02% NaN_3, pH 7.8) containing 0.5% Triton X-100 or 0.25% CHAPS and supplemented with enzyme inhibitor Complete™ (Boehringer-Mannhiem). Following centrifugation (10,000×*g*, 10 min), solubilized proteins were analyzed by either immunoprecipitation or immunoabsorption as described previously (19, 29).

Western blotting was carried out using a modification of previously described methods (30, 31). Cells (at 10^8/ml) were solubilised by incubation (1 h, ice) of cells in lysis buffer containing 0.5% Triton X-100 , 0.25% CHAPS and the enzyme inhibitor mix, Complete™ (Boehringer). Following centrifugation (10,000×*g*, 10 min), lysates ($1–2\times10^6$ cell equivalents) were fractionated on a 7.5% polyacrylamide gel (Mini Protean II, Biorad, Hercules, CA, USA) under reducing conditions (5% B-mercaptoethanol) and separated proteins transferred to nitrocellulose (NC; HybondC, Amersham, Buckinghamshire, UK) as described previously (19, 29). The membranes were incubated (37°C) overnight (o/n) in PBS, then blocked for 1 h with 5% non-fat dry milk powder (MP) in PBS. All subsequent steps were carried out using ice cold solutions. NC was incubated o/n with mAb solution containing 1% MP. Following incubation, NC was washed in PBS then incubated for 15 min with 0.25% glutaraldehyde/PBS, prior to washing (15 min) sequentially with 0.1 M glycine in PBS (pH 8.5), 0.1% BSA/PBS, 1% MP/PBS and 0.1% goat serum (GS)/PBS. Following washing, NC was incubated 1 h with GAM-Biotin (Dako 1:1000 in 10% GS/PBS) then washed in PBS and incubated (1 h) with streptavidin-HRP (Amersham) diluted in 1% BSA/PBS. Reactive protein bands were then visualized by chemiluminescence using Super Signal (Pierce, Rockford, IL, USA). The molecular weight of visualized bands was determined by comparison with biotinylated MW standards (Biorad).

Assessment of temperature induced changes in mAb binding and localisation

Temperature induced changes in mAb binding and/or localisation were assessed using flow cytometry. Unless stated otherwise, all steps were carried out on ice using ice-cold solutions for washing and incubations.

a) Changes in the density of cell surface associated mAb were determined by labelling cell aliquots as described above. Samples were then washed, resuspended in PBS and aliquots incubated for fixed times, either on ice or at 37°C. The 37°C samples were


A. Blood leukocytes:
i) granulocytes
ii) ER - PBMC
iii) ER+ PBMC
Activated
Fresh
Control
CMRF-56
CD14,16 & 19
CD 3
B. T + PMA/Cal
CD 3-PE
CD 83
CMRF-44
C. Non T cultured
CD 19-PE
D. Non T + IFNγ
CD 14-PE
E. Tonsil non T

then returned to ice and surface bound antibody detected with FITC-SAM. For each treatment the MFI (FITC channel) of the 37°C sample was expressed as a percentage of the corresponding ice sample MFI (37°C/ice×100).

b) Antibody internalization was assessed using fluorochrome (FITC or PE)-conjugated mAb (F1-mAb). Cells were labelled with F1-mAb, then washed, resuspended in PBS and aliquots incubated either on ice or 37°C. The 37°C samples were then placed back on ice and the remaining surface associated F1-mAb detected by labelling with PE-SAM or FITC-SAM (F2). The MFI of the F1-mAb complex was used as a measure of the total surface and internalized mAb and the MFI of the fluorochrome-SAM (F2) as a measure of the remaining surface-bound mAb. For each treatment (and fluorochrome) the MFI of the 37°C sample was expressed as a percentage of the corresponding ice sample MFI (37°C/ice×100). Data from triplicate samples are shown as mean percentage (SEM.

c) The temperature dependence of changes in surface-bound mAb levels was assessed using FITC-conjugated mAb. Cell aliquots were incubated 1 h (ice) with FITC-mAb. Samples were then split and the aliquots (still containing cells and FITC-mAb solution) were incubated 5 min on either ice or at 37°C, before the 37°C samples were returned to ice. These aliquots (ice and 37°C) were then split again and then washed and fixed either immediately (t= 0) or after a further 60 min (t=60) incubation on ice. Data was expressed as MFI, normalized relative to the ice sample at t=0 (100%).

Results

Generation of the CMRF-56 mAb

Following immunization with the HD-derived cell line L428, hybridomas producing mAb reactive with L428 but not PBMC were identified, then analyzed for reactivity with the low density (DC-enriched) fraction of cultured PBMC. The mAb CMRF-56 (IgG_1) labelled the DC within these preparations and was characterized as described below.

CMRF-56 reactivity with normal haemopoietic non-DC populations

Cell surface expression of the CMRF-56 antigen on isolated blood and tonsil leukocyte populations was analyzed by both single and double labelling in conjunction with flow cytometry. The CMRF-56 mAb did not react with circulating PBMC (n=5), peripheral blood granulocytes, FMLP-activated granulocytes (n=3, Fig. 1A), the $CD16^+$, $CD14^+$ and $CD19^+$ populations within ER^- PBMC preparations (n=6, Fig. 1A) or the $CD3^+$ population within ER^+ PBMC preparations (n=4, Fig. 1A).

In vitro culture (37°C) of ER^+ PBMC (n=3) for 24 h and 72 h in medium (10% FCS/RPMI) or medium supplemented with PMA/CaI (Fig. 1B) or PHA (n=3) did not induce CMRF-56 antigen expression on the CD3+ population. Similarly, T lymphocytes activated with solid phase CD8 mAb or CD3 mAb plus IL-2 were also $CMRF\text{-}56^-$ (data not shown). Culture of ER^- PBMC in medium (16 h, 37°C) induced the expression of the CMRF-56, CD83 and CMRF-44 antigens on subpopulations of the $CD19^+$ lymphocytes (Fig. 1C), whereas the $CD19^-$ population (including $CD14^+$ monocytes) did not express the CMRF-56 antigen. The culture (48 h) of ER^- and ER+ PBMC preparations in the presence of PMA/CaI induced CMRF-56 and CD83 expression on the majority of $CD19^+$ cells present but did not induce expression of these antigens on the $CD19^-$ cells (data not shown). Culture of ER^- PBMC for 24 h and 72 h in medium supplemented with additional IFNγ (Fig. 1D), LPS, GM-CSF or TNFα (data not shown) failed to induce the expression of CMRF-56 on the CD14+ monocyte population despite the induction of changes in CMRF-44, CD71 or MHC class II antigen expression (n=3).

Analysis by flow cytometry of isolated tonsillar lymphocytes confirmed that CMRF-56 did not label tonsil T lymphocytes (n=4) but, in common with CD83 and CMRF-44, did label a proportion of the tonsil B lymphocytes with moderate intensity (Fig. 1E).

Fig. 1. CMRF-56 reactivity with blood and tonsil leukocytes. **(A): (i) Fluorescent intensity histogram of freshly isolated and and FMLP activated granulocytes stained with CMRF-56 or isotype control (filled); (ii) dot plots of ER^- PBMC double labelled with CMRF-56 vs. CD3, CD14, CD16, CD19-PE; and (iii) dot plot of ER^+PBMC double labelled with CMRF-56 vs CD3-PE. (B) Dot plots of PMA/CaI activated (72 h) ER^+PBMC, double labelled with the mAb indicated. (C) Dot plots of cultured (16 h, 37°C) ER^- PBMC double labelled with the mAb indicated and gated according to size on the lymphocyte population. (D) Dot plots of IFNγ activated (24 h) ER^- PBMC double labelled with the mAb indicated and gated according to size on the monocytoid population. (E) Dot plots of tonsil ER^- lymphocytes double labelled with the mAb indicated. In all cases the gates delineating positive staining shown were set on the basis of isotype matched negative control staining. Data are from representative experiments of several performed (see Results).**

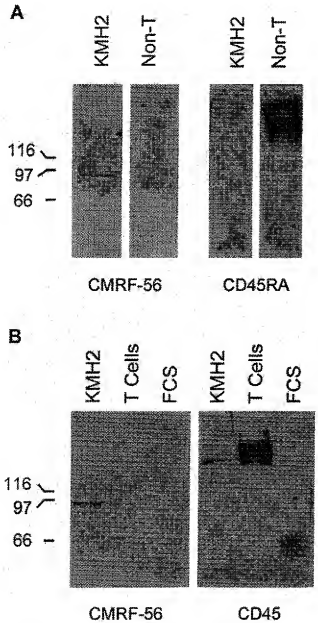


Fig. 2. **Western blot analysis of the CMRF-56 antigen. Whole cell lysates of ER$^-$ PBMC, ER$^+$ PBMC and the cell line KMH2 were analyzed, together with FCS (10 μg) for the presence of a CMRF-56-specific antigen as described in Material and methods. Blots from each gel (A, B) were stained with either CMRF-56 or the isotype-matched mAb CMRF-11 (CD45RA) and CMRF-26 (CD45). The position of MW markers (kDa) are shown on the left. Two separate Western blots representative of four experiments are shown.**

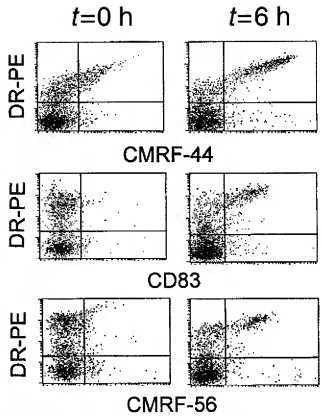


Fig. 3. **CMRF-56 antigen expression is inducible on directly isolated DC. (A) Directly isolated blood DC were cultured in medium for 0 or 6 h then analyzed by double labelling with CMRF-56, CMRF-44 or CD83 vs DR-PE. In all cases the gates delineating positive staining shown were set on the basis of negative control staining. Data are from a representative experiment of three performed.**

In all tonsil B lymphocyte preparations analyzed (n=5) there was a clear difference in the percentage of B lymphocytes labelled with these mAb: the CMRF-44 antigen was expressed on a higher percentage and the CD83 antigen on a lower percentage of cells than the CMRF-56 antigen.

Reactivity with cell lines and transfectants

The cell surface expression of CMRF-56 antigen on human cell lines was analyzed by flow cytometry. The CMRF-56 antigen was strongly expressed (change in MFI >50) on a number of B-cell-(Mann, Raji) and HD-derived (L428, KM-H2, HDLM-2) cell lines but was not expressed at detectable levels by the myelo-erythroid K562 line, the T-lymphoid lines HSB2 and Molt 4, the myeloid leukemia cell lines NB4, THP1, U937, KG1 and KG1a or the pre-B-lymphoid line NALM6 (n=3, data not shown). The CMRF-56 mAb was similarly unreactive with the CD83$^+$ T-lymphoid cell line Jurkat and CD83-positive COS cell transfectants (n=3, data not shown). In all cases CMRF-56 staining was homogeneous and either 0% or 100% of cells were positive.

Biochemical analysis

Immunostaining of L428 detergent lysates demonstrated that the CMRF-56 antigen was effectively solubilised by the non-ionic detergent Triton X-100 and the zwitteronic detergent CHAPS. Immunoprecipitation experiments using lysates of cell surface or metabolically-labelled cell lines failed to identify the CMRF-56 antigen despite co-precipitation of appropriate molecular weight products with MHC class II and CD83 reagents (data not shown). Western blotting detected a specific 95 kDa band in the lysates of the cell line KMH2 which was absent in lysates prepared from the T or non-T fractions of PBMC (Fig. 2).

CMRF-56 reactivity with isolated DC

The reactivity of CMRF-56 with isolated DC populations was examined by indirect immunofluorescence and flow cytometry. Directly isolated fresh blood DC (Fig. 3) did not express detectable levels of either the CMRF-56 or CD83 antigens. However expression of both antigens was rapidly induced on directly isolated DC within 6 h of *in vitro* culture. In contrast, expression of the CMRF-44 antigen was consistently detected on a subpopulation of directly isolated blood DC and further upregulation of the CMRF-44 antigen preceded that of both the CMRF-56 and CD83 antigens.

Analysis of the DC-enriched low-density fraction of cultured

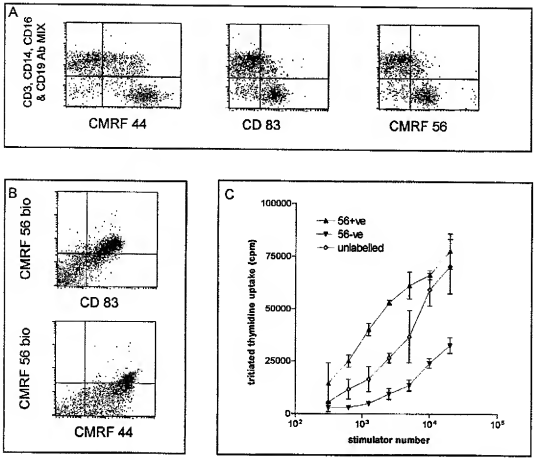


Fig. 4. Analysis of CMRF-56 antigen expression within cultured low density preparations of ER$^-$ PBMC. **(A)** Dot plots of preparations double labelled with CMRF-44, CD83 or CMRF-56 vs. a mix of PE-conjugated CD3, CD14, CD16 and CD19 mAb. **(B)** Dot plots of preparations double labelled with CD83 or CMRF-44 vs. CMRF-56-biotin. In all cases the gates delineating positive staining shown were set on the basis of negative control staining. **(C)** Allogeneic MLR performed following sorting of a low-density preparation on the basis of CMRF-56 expression. The CMRF-56-positive (Δ) and -negative (▽) populations together with unsorted controls (◇) were cultured with allogeneic T-lymphocytes for 5 days then (^{3}H) TdR incorporation determined. Results are expressed as the mean of triplicate counts ±SEM. Data are from a representative experiment of three performed.

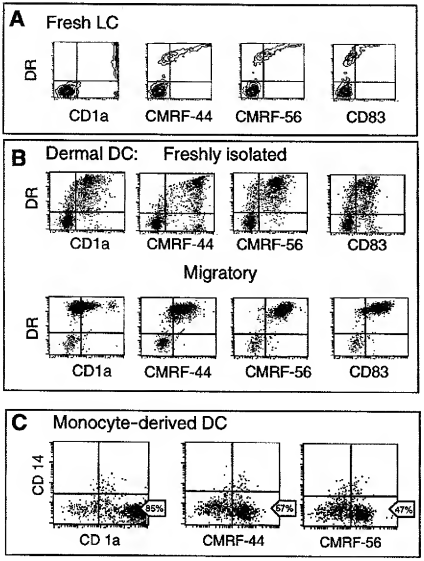


Fig. 5. **CMRF-56 reactivity with (A) fresh LC, (B) fresh dermal DC and migratory dermal DC and (C) *In vitro* generated DC.** Preparations were double labelled with the mAb indicated and the gates delineating positive staining set on the basis of negative control staining. Data are from representative experiments of three performed with each type of cell preparation.

ER^- PBMC consistently identified a subpopulation of $CMRF\text{-}56^+$ cells (10–30%, $n=20$) identical to the DC populations detected by CD83 and CMRF-44 (Fig. 4A). Double labelling (Fig. 4B) confirmed that the CMRF-56 reactivity was associated with the lin^-, $CMRF\text{-}44^+$ and $CD83^+$ DC populations. FACS sorting of low density ER^- PBMC on the basis of CMRF-56 expression clearly demonstrated that potent allostimulatory activity was associated with the $CMRF\text{-}56^+$ population and that the $CMRF\text{-}56^-$ population was only weakly stimulatory ($n=3$, Fig. 4C). Binding of the mAb did not affect the DC allostimulatory activity (data not shown).

Freshly-isolated $CD1a^+$ LC ($n=3$) express the CMRF-56 and CMRF-44 antigens at high density, whilst CD83 expression was considerably weaker (Fig. 5A). Likewise, fresh dermal DC ($n=3$) were strongly CMRF-44-positive, expressed CMRF-56 at moderate levels and showed relatively weak expression of CD83 (Fig. 5A). Migratory dermal DC, which have been exposed to 48 h *in vitro* culture during preparation expressed moderate levels of all 3 antigens (Fig. 5A).

In vitro generated Mo-DC populations were also studied ($n=3$). Following culture of monocytes in the presence of GM-CSF, IL-4 and TNFα, the resulting Mo-DC were strongly $CMRF\text{-}56^+$. The percentage of $CMRF\text{-}56^+$ cells was significantly less than the percentage of CD1a+ cells (Fig. 5B).

Heterogeneous expression of HLA-DR has been observed on both directly isolated SF DC and tonsil DC. The majority of isolated SF-DC and tonsil DC did not express either the CMRF-44, CMRF-56 or CD83 antigens ($n=5$, Fig. 6). However, the small subpopulations of these DC

preparations expressing HLA-DR at relatively high density (DR^{high}) coexpressed moderate levels of the CMRF-44 antigen, lower levels of the CMRF-56 antigen and negligible CD83. Following culture, further upregulation of these antigens was observed with CMRF-44 antigen induction preceding that of CMRF-56 and CD83 ($n=5$).

Immunohistological analysis of CMRF-56 expression

Immunohistological staining of lymph node and tonsil sections detected weak CMRF-56 antigen expression on the germinal centre lymphocytes but high density expression on scattered cells within the interfollicular (T-cell) zones. Immunofluorescent double labelling of tonsil sections demonstrated that the CMRF-56-positive interfollicular cells lacked CD19 and CD20 but the majority co-expressed CD83, although some only express low levels of the CD83 antigen (Fig. 7).

Temperature-induced changes in mAb binding and/or localization

In preliminary experiments it was observed that incubation of CMRF-56-labelled cells (KM-H2, Raji, L428, Mo-DC) at 37°C resulted in a significant decrease in the amount of surface bound mAb relative to cells incubated on ice. This decrease in surface-bound CMRF-56 was rapid, with a significant reduction being observed within 5 min of incubation (40–80%, $n=20$) and little further reduction being observed over longer periods of incubation (5–30 min, data not shown). Comparison with other mAb demonstrated that the level of cell surface CMRF-44 binding was similarly rapidly diminished by 37°C incubation, whereas the level of CD83 and CD40 mAb binding was only minimally reduced (Fig 8A, data not shown).

The possibility that 37°C incubation of CMRF-56- and CMRF-44-labelled cells results in mAb internalization was addressed by label-

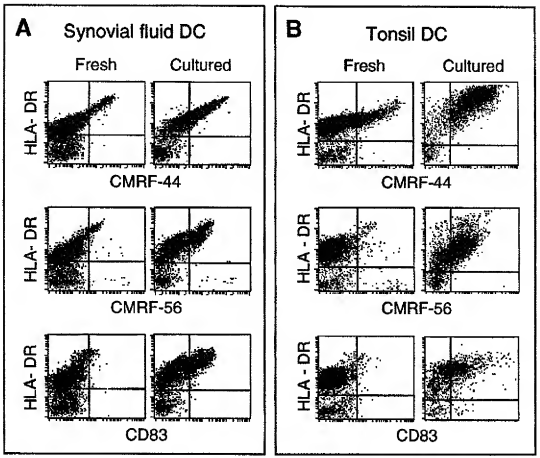


Fig. 6. CMRF-56 and CD83 reactivity with isolated SF-DC and tonsil DC, before and after *in vitro* culture. Preparations of (A) SF-DC and (B) Tonsil DC were double labelled as indicated, before and after 16 h culture in medium. In all cases gates delineating positive staining were set on the basis of negative control staining. Data are from representative experiments of five performed on each type of DC preparation.

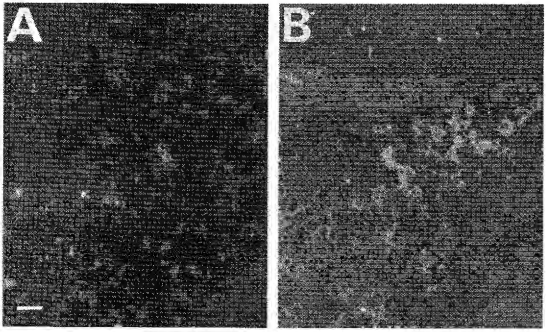


Fig. 7. **CMRF-56 staining of tonsil tissue as visualized by dual-color immunofluorescence. (A) CMRF-56$^+$ cells (green) present in the interfollicular T-cell-enriched areas do not express CD19 and CD20 (red). (B) The majority of CMRF-56 cells (green) co-express CD83 (red).** Magnification 286×. Bar size=25 μm.

ing cells with fluorochrome-conjugated mAb (F1) and then, following incubation, detecting remaining cell surface associated mAb by labelling with SAM-conjugated to a second fluorochrome (F2). As shown in Fig. 8A incubation of CMRF-56- or CMRF-44-labelled cells results in similar decreases of both the total (F1) and cell surface associated (F2) MFI, indicating that these mAb were not internalized. Incubation of cells labeled with CD71mAb specific for the transferrin receptor resulted in a decrease of surface bound mAb whilst the total mAb level remained unchanged, which is in accordance with the well-documented internalization of transferrin receptor-ligand complexes at 37°C (32).

In order to determine whether the decrease in CMRF-56 and CMRF-44 was a reversible temperature-dependent process, further experiments were performed using FITC-conjugated mAb. In this system, cells were labelled on ice with FITC-conjugated mAb, then incubated (ice or 37°C) in the continued presence of FITC-conjugated mAb for 5 min, then washed and fixed either immediately (t=0) or after reincubation for a further 60 min on ice to allow the possible re-establishment of mAb binding. As shown in Fig. 8B, the incubation at 37°C of CMRF-56-labelled cells, in the continued presence of mAb, still resulted in decreased mAb binding. The level of CMRF-56 mAb binding was, however, significantly restored by reincubating the 37°C treated samples on ice, demonstrating that the level of CMRF-56 binding is a reversible temperature dependent process. In contrast, the decrease in CMRF-44 binding induced at 37°C was not reversed by reincubation of cells with mAb on ice.

The observation that incubation at 37°C in the presence of mAb still resulted in decreased CMRF-56 binding demonstrates that CMRF-56 antigen synthesis and re-expression does not explain the rebinding of CMRF-56 that is observed at 4°C.

Discussion

The mAb CMRF-56 has been shown to recognize a previously undescribed 95 kDa DC activation antigen with limited expression on other haemopoietic populations. Circulating blood leukocytes did not express the CMRF-56 antigen and, following either *in vitro* culture or activation of PBMC populations, expression of the CMRF-56 antigen was detected only on a subset of the low-density (DC-enriched) fraction of cultured PBMC and on a subpopulation of CD19$^+$ lymphocytes. FACS sorting of the CMRF-56$^+$ cells present in the low density fraction of cultured PBMC confirmed that these cells possess the functional attributes of DC. The finding that circulating blood DC are CMRF-56$^-$ but induce high-density antigen expression within 6-h culture confirmed that CMRF-56 recognizes a early differentiation/ activation marker on DC.

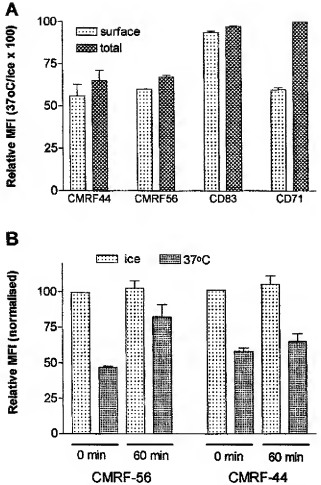


Fig. 8. **Temperature induced changes in cell associated CMRF-56, CMRF-44 and CD83 mAb binding and localisation. (A) Antibody internalization.** The cell line KMH2 was labelled with FITC-conjugated (CMRF-56, CMRF-44, CD71) or PE-conjugated (CD83) mAb(F1-mAb), then samples split and incubated 5 min on ice or 37°C before detection of surface bound mAb with SAM-PE or SAM-FITC (F2). The MFI of the mAb (F1-mAb) was used as a measure of total mAb level (surface + internalized) and the MFI of the SAM reagent (F2) as a measure of surface-bound mAb. Triplicate samples of each mAb were analyzed and for each the MFI of the 37°C samples was expressed as a percentage of the corresponding ice sample MFI. Data are shown as mean percentage±SEM and are from a representative experiment of five performed. **B) Temperature dependence of changes in mAb binding.** This was measured by labelling KMH2 with FITC-conjugated CMRF-44, CMRF-56, CD83 or CD71, then splitting and incubating (5 min) the cells at 37°C or on ice in the continued presence of FITC-mAb. Samples were then washed and fixed either immediately ($t=0$) or following a further 60 min incubation on ice ($t=60$). The MFI of each treated sample ($n=3$) was expressed as a percentage normalized relative to the ice, $t=0$ sample (100%). Data are shown as the mean+SEM of triplicate samples and are from a representative experiment of four performed.

At present, the only selective DC surface markers available are the CMRF-44 (15, 19) and CD83 (20, 21) antigens, which are also early activation markers on DC. The CMRF-44 antigen, but not the CD83 and CMRF-56 antigens, is expressed on a subpopulation of circulating DC (15), with all three markers being rapidly upregulated with culture. Isolated SF-DC and tonsil DC preparations lack CD83 expression but appear to contain a more activated subpopulation of DC than those observed in blood as both CMRF-56- and CMRF-44-positive subpopulations were detected. The expression of all three antigens within these preparations was again rapidly upregulated with culture. This, together with the pattern of CMRF-56, CMRF-44 and CD83 antigen expression and induction on Mo-DC, skin DC and B-lymphocyte populations suggests that these three distinct antigens upregulate rapidly in the order of the CMRF-44 antigen followed by the CMRF-56 antigen and then the CD83 antigen. In addition, to these apparent kinetic differences these three DC differentiation/activation antigens can be clearly distinguished on the basis of their differing reactivity with activated non-DC leukocytes, cell lines and transfectants. The demonstration by Western blotting that CMRF-56 specifically recognizes a 95 kDa antigen further distinguishes it from CD83 which is a 45 kDa glycoprotein (20).

The slower induction of CD83 antigen expression may be due to the cleavage of surface protein and release of soluble CD83 which has been reported to occur with activated B lymphocytes (34). We have confirmed that soluble CD83 is shed from human cell lines in tissue culture (data not shown). Thus it is theoretically possible that proteolysis and/or antigen shedding reduces the expression of CD83 on certain isolated DC populations.The pattern of CMRF-56 expression on human cell lines parallels that of the CMRF-44 and CD83 antigens in many respects, being restricted to HD derived and B-cell lines whereas cell lines of myeloid and T lymphoid origin lack or only weakly express these antigens. A similar pattern is observed following activation of ER$^-$ PBMC or ER+ PBMC populations, with expression of these antigens on non-DC being most readily inducible on B lymphocytes.

Although CMRF-44 antigen expression can be induced on CD14$^+$ monocyte populations using a range of single stimuli (19), negligible expression of the CMRF-56 and CD83 antigens occurs under these conditions. Nonetheless, CD83 expression can be induced on cells of myeloid origin following long-term culture in the presence of particular cytokine combinations (10). These cells also express the CMRF-44 antigen (9) and closely resemble DC in terms of function and phenotype. As shown in this study, these *in vitro*-derived Mo-DC also expressed the CMRF-56 antigen.

It is clear that the specificity of the CMRF-56 and CD83 mAb for DC populations is not absolute. Nevertheless, the study of these antigens in conjunction with B lymphocyte markers provides a

highly selective means of identifying DC populations at an early stage of activation, both *in situ* and within isolated leukocyte populations. The upregulation of these molecules is associated with a phase of significant activation of DC function. Thus, DC upregulate the costimulator molecules CD80, CD86 (12, 13, 35), CD40 (23) together with adhesion molecules such as ICAM-1 (36), in parallel with the changes described here. As yet no definite function for the antigens CMRF-44, CMRF-56 or CD83 has been identified although functional studies have been to date limited to analyzing the effect of adding mAb to the allogeneic MLR. It is perhaps significant that strong CMRF-56, CMRF-44 and CD83 labelling of both cell lines and activated leukocytes is not restricted to a particular lineage but is associated with strong allostimulatory activity.

The interaction of ligands with specific cell surface antigens can induce modulation of the level of antigen expression. The ligation of a number of leukocyte antigens with mAb has been shown to induce subsequent antigen internalization (37–41) or shedding (42–47). We therefore examined whether ligation of the DC restricted antigens CMRF-56, CMRF-44 and CD83 with mAb results in antigen modulation as this would potentially provide evidence relevant to their function. As demonstrated in this study, the binding of these mAb did not trigger Ag internalization at 37°C. However, the amount of CMRF-56 and CMRF-44 mAb bound at the cell surface was significantly and rapidly reduced by incubation at 37°C. Potential explanations for this effect include antigen shedding (42–47), a decrease in mAb affinity at 37°C (48) or a conformational change in the antigen after monoclonal antibody binding at 37°C (49).

The decrease in the observed CMRF-56 mAb binding at 37°C can be significantly (but not totally) reversed by returning the cells to 4°C. Therefore the majority of this effect is probably due to either a reversible temperature dependent conformational change in the CMRF-56 antigen or is due to the CMRF-56 mAb itself having a lower affinity at 37°C. In contrast, the decrease observed with the CMRF-44 mAb was not due to internalization and was not reversible by reincubation with excess mAb on ice. This strongly suggests that the CMRF-44 antigen undergoes antigenic shedding or an irreversible conformational change following ligand binding and incubation at 37°C. We were unable in this study to detect any significant decrease in CD83 mAb when incubated for 5–30 min at 37°C despite the fact that soluble CD83 is detectable by ELISA in cell line conditioned medium (in preparation). Despite this the methods utilized in this study do provide a relatively simple and rapid method of screening mAb for the ability to induce dramatic changes in cell surface antigen conformation or density following ligation.

Although the function of the CMRF-56 antigen is at present unknown, the results of this study identify CMRF-56 as a valuable new marker of human DC populations. The CMRF-56 mAb has a different pattern of reactivity to that of the previously described DC markers CMRF-44 and CD83 and therefore provides a useful additional tool in the study of DC. However, its specificity, just like that of CMRF-44 and CD83 mAb, is not absolute and care should be exercised in applying these mAb to the analysis of activated cell populations, particularly with potent signal inducers such as complex cytokine mixtures or PMA/Cal. For this reason, we are continuing attempts to generate mAb to resting lin$^-$ blood DC.

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EXHIBIT 2

CMRF-56 Immunoselected Blood Dendritic Cell Preparations Activated With GM-CSF Induce Potent Antimyeloma Cytotoxic T-cell Responses

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Summary: The efficient antigen-presenting function of dendritic cells (DC) makes them an attractive cellular adjuvant for clinical immunotherapeutic protocols aimed at eradicating minimal residual disease after conventional treatment of multiple myeloma (MM) and other malignancies. We used single-step positive immunoselection with biotinylated CMRF-56 monoclonal antibody to generate a $CD11c^+$ blood DC (BDC) enriched antigen-presenting cell population, which, after exposure to activation stimuli for as little as 2 hours, displayed a mature costimulatory BDC phenotype and secreted inflammatory cytokines. Of the activation stimuli tested, granulocyte macrophage colony-stimulating factor (GM-CSF) provided optimal activation of the CMRF-56 immunoselected preparations and primed efficient cytotoxic T cell (CTL) responses using MART-1 peptide as a model tumor-associated antigen. In addition, GM-CSF activated CMRF-56 immunoselected cells cross-presented MM cell lysate and improved the MM-specific polyclonal CTL response (no activation $18.8\% \pm 4.3\%$ vs. GM-CSF activation $40.9\% \pm 7.3\%$, $P = 0.051$). CMRF-56 immunoselected BDC migrated in vitro both spontaneously and specifically toward the secondary lymphoid chemokine CCL21. Their migration was also significantly improved by GM-CSF and prostaglandin E_2 activation and a greater percentage of activated BDC migrated specifically compared with monocyte-derived DC. These results indicate that the CMRF-56 immunoselected BDC preparations can cross-present antigen for effective anti-MM CTL responses and that limited exposure to maturation stimuli can produce phenotypically and functionally mature migrating DC. CMRF-56 immunoselected cells are suitable for use as part of an immunotherapeutic anti-MM vaccine.

Key Words: cellular immunotherapy, dendritic cells, multiple myeloma, cytotoxic T cells

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Dendritic cells (DC) are potent antigen-presenting cells that link the innate and adaptive immune responses.[1] Given the potential of tumor immunotherapy, there has been escalating interest in the biology of DC in cancer patients and their potential to induce responses against various tumors including multiple myeloma (MM). The results of DC vaccination trials have given some encouragement[2] but there are considerable opportunities to improve this approach, particularly in MM, a disease in which recent therapeutic advances have controlled the disease, but have not eradicated it. MM is susceptible to allogeneic effectors[3] but attempts to exploit DC immunotherapy in MM have yet to be proven efficacious.[4] The DC preparation to be used must be further researched and optimized[5] to identify the best DC preparation, its optimal maturation status, an efficient antigen loading method, and maximization of their migratory capacity.

DC may be generated in vitro from isolated monocytes using interleukin (IL)-4 and granulocyte macrophage colony-stimulating factor (GM-CSF) to differentiate the DC [monocyte-derived DC (MoDC)], or from $CD34^+$ hematopoietic stem cells derived from cord blood or mobilized peripheral blood ($CD34^+$-derived DC). These "laboratory manufactured" cells acquire characteristics dictated by the culture conditions used and there are logistic and regulatory issues of consequence given the extensive in vitro culture required. An alternative is to use preformed, "patient manufactured" blood DC (BDC) either the myeloid ($CD123^{lo}$ $CD11c^+$) or plasmacytoid ($CD123^{hi}$ $CD11c^-$) subsets circulating in the patient. It is possible to obtain these cells in sufficient quantities from blood, without the need for extended in vitro culture periods or cytokine supplementation.[6–8] Furthermore, single-step positive immunoselection, with biotinylated CMRF-56 monoclonal antibody (mAb), can be used to recover BDC from overnight cultured peripheral blood mononuclear cells (PBMCs). The CMRF-56 antigen is expressed by BDC after in vitro culture for as little as 6 hours and follows kinetics akin to but distinct from the DC differentiation/activation markers CD83 and CMRF-44.[7,9] Comparisons among $CD34^+$ DC, MoDC, and BDC have revealed morphologic, molecular, and functional differences, however, their relevance is unclear. For example, BDC express higher levels of major histocompatibility complex and costimulatory molecules,[6,10] induce more T_H1 effector cells than MoDC,[11] and migrate toward secondary

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lymphoid chemokine CCL21 without the need for activation.[12] BDC also generate equivalent peptide-specific cytotoxic T cell (CTL) responses to MoDC.[13] These differences have yet to translate into documented differences in clinical efficacy, but it is of interest that gradient purified antigen-presenting cell preparations have produced well documented responses in low-grade lymphoma[14] and prostate cancer.[15] As CTL induction seems to be a critical component of an effective anticancer response,[16] we saw it as essential to study the ability of CMRF-56 selected BDC to process and cross-present antigen to CTL.

After exposure to pathogen-specific molecular patterns, proinflammatory cytokines and T-cell–derived signals, DC undergo maturation/differentiation/activation resulting in down-regulated antigen uptake, increased antigen processing and presentation, induction of costimulatory molecules, secretion of chemokines and cytokines, and altered migration. Reduplicating this process for clinical MoDC preparations has been necessary to induce antitumor immune responses as immature DC can induce antigen-specific T-cell tolerance.[17] Activation of MoDC is also essential to improve their limited migration capacity.[18] A variety of DC activation stimuli have been examined for their ability to induce DC maturation and migration including Toll-like receptor (TLR) ligands polyI:C[19–22] and imiquimod,[23–25] CD40 ligand,[26,27] GM-CSF,[28,29] and the cytokine cocktail [tumor necrosis factor (TNF)-α, IL-6, IL-1β, and prostaglandin E_2 (PGE_2)] commonly used to induce MoDC maturation.[30,31] These activation stimuli have been widely investigated in MoDC and in limited preliminary in vitro studies using BDC.[10,13] Although there is a clear consensus that activation is important for MoDC function, it is unclear whether activation of BDC is necessary and, if so, which activation stimulus may be optimal.

CMRF-56 immunoselected preparations have been shown to up-regulate BDC costimulatory molecule expression, prime CD4T cell responses (keyhole limpet hemocyanin and tetanus toxoid proteins) and induce peptide-specific CTL responses to control antigens.[7,32] This study provides the first detailed analysis of the cellular constitution of the CMRF-56 immunoselected cell preparation. It also tested the hypothesis that maturation/differentiation/activation of the CMRF-56 immunoselected BDC might improve their ability to process and cross-present intact MM antigen for CTL priming in vitro. Further, it established that the maturation/differentiation/activation of BDC enhanced their migratory capacity and that these preparations had greater migratory capacity than MoDC.

MATERIALS AND METHODS

Cell Sources

Peripheral blood and leukapheresis products were obtained from healthy volunteers under protocols approved by the Mater Misericordiae Hospitals Ethics Committee. PBMCs were separated by gradient centrifugation using 10 mL Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden) to underlay 40-mL blood product diluted 1:3 with phosphate-buffered saline (PBS). PBMCs were collected from the interface and washed in PBS to remove contaminating platelets before culture. The U266 (myeloma, HLA-$A2^+$), SKMEL05 (melanoma, HLA-$A2^+$), SKMEL28 (melanoma, HLA-$A2^-$), and K562 [natural killer (NK) cell target, HLA$^-$] cell lines were obtained from American Tissue Culture Collection and documented, Mater Medical Research Institute (MMRI) mycoplasma free stocks were used.

CMRF-56 Immunoselection

PBMCs were cultured for 14 hours in complete RPMI-1640 media (Gibco-Invitrogen, Mount Waverley, VIC, Australia) containing 10% AB serum (Australian Red Cross Blood Service), HEPES buffer, sodium pyruvate, 2-mercaptoethanol, L-glutamine, and nonessential amino acids (Gibco-Invitrogen) at 10×10^6/mL to up-regulate the CMRF-56 antigen.[9] Cells were removed from the culture dish with cold PBS, and labeled with 10 μg/mL biotinylated CMRF-56 antibody (MMRI, Brisbane, Queensland, Australia) at 66×10^6 cells/mL for 15 minutes at 4°C. Antibiotin microbeads (Miltenyi Biotech, Gladbach, Germany) were added at 100 μL/100×10^6 cells, and incubated for 20 minutes at 4°C. CMRF-56–positive cells were immunoselected using VarioMACS LS columns (Miltenyi Biotech). Samples of cells were taken from those loaded on the column, positively selected and not selected (negative fraction) and analyzed by flow cytometry to calculate the BDC purity, yield, and recovery.

Monocyte-derived DC Preparation

$CD14^+$ monocytes were selected from PBMC by positive magnetic immunoselection using anti-CD14 antibody and goat-antimouse IgG microbeads (Miltenyi). The cells were cultured with 800 U/mL GM-CSF (PeproTech, Rocky Hill, NJ) and 1000 U/mL IL-4 (PeproTech, Rocky Hill, NJ) for 5 days, and activated where indicated with the cytokine cocktail (see section Activation Stimuli) for a further 24 hours before the assays were performed.

Activation Stimuli

Several clinically relevant DC activation stimuli were tested for their ability to activate CMRF-56^+ cells. The TLR ligands polyI:C and imiquimod (InvivoGen, San Diego, CA) were used at 50 μg/mL and 10 μg/mL, respectively. Soluble CD40L (Amgen, Thousand Oaks, CA) was used at 1 μg/mL. The cytokine cocktail consisted of 5 ng/mL IL-1β, 5 ng/mL TNF-α, 150 ng/mL IL-6 (R & D Systems, Minneapolis, MN), and 1 μg/ml PGE_2 (Sigma-Aldrich, Castle Hill, NSW, Australia). GM-CSF (PeproTech, Rocky Hill, NJ) was used at 1000 U/mL.

Fluorescent Antibodies and Flow Cytometry

Fluorescently labeled antibodies defining cell lineage and purity of CMRF-56 immunoselected cells included CD3, 4, 8, 14, 19, 20, 56 and HLA-DR

(BD Biosciences, North Ryde, NSW, Australia), CMRF-56 (MMRI, Brisbane, QLD, Australia), and Streptavidin-PC5 (Dako Cytomation, Botany, NSW, Australia). DC subsets were defined using CD11c, 16, 34, 56 (BD Biosciences), BDCA1, and BDCA3 (Miltenyi Biotech). DC activation was assessed using CD40, 80, 83, and 86 (BD Biosciences). MART-1 CTL induction was evaluated on live cells defined as 7AAD negative (BD Biosciences) and MART-1 pentamer (ProImmune, Oxford, UK) positive. Cells stained with 7AAD were analyzed within 3 hours of staining, whereas other samples were fixed with 1% paraformaldehyde (Sigma). Data were acquired on FACS Calibur (BD Biosciences) and analysis performed with FCS Express 2 software.

The Cellular Constitution of CMRF-56 Immunoselected Cells

CMRF-56 immunoselected cells were labeled with various discriminatory lineage antibodies and analyzed by flow cytometry. BDC were defined as lineage (CD3, CD14, CD19, CD20, and CD56) negative, HLA-DR–positive cells. The percentage of each cell type was calculated as a proportion of the immunoselected population, and in each case, totals 100% (Fig. 1).

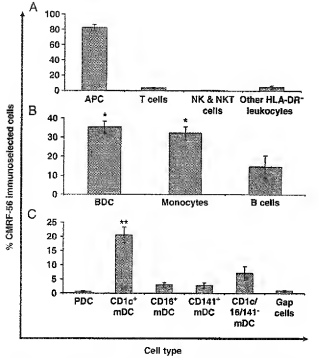

FIGURE 1. Cellular constitution of the CMRF-56 immunoselected preparation. CMRF-56 immunoselected cells were analyzed by flow cytometry to identify constituent cell populations. A, Subdivision of major cell types. B, Types of APC present. C, Analysis of BDC subsets. APC indicates antigen-presenting cell; NKT, natural killer T cell; mDC, myeloid dendritic cell; PDC, plasmacytoid dendritic cell, gap cells = $CD123^-CD11c^-$ DC. $P<0.05$ (*) and $P<0.01$ (**).

Analysis of Mature BDC Phenotype and Cytokine Production

Immediately after immunoselection, a sample of the CMRF-56 immunoselected cells was analyzed by flow cytometry to document baseline expression of the costimulatory molecules CD83, CD80, CD40, and CD86. After exposure of individual cultures of CMRF-56 cells to the defined activation stimulus for a 2-hour period, the cells were washed and recultured at 1×10^6/mL for a further 22 hours. Supernatants were collected for analysis of secreted cytokines and stored at −80°C until analysis by flow cytometry using the BD Cytokine Bead Array Human Inflammation Kit. The CMRF-56 immunoselected cells were harvested and their expression of costimulatory molecules analyzed by flow cytometry.

Migration Assay

After incubation with GM-CSF, GM-CSF and PGE_2, the cytokine cocktail, or no activation stimulus for 2 hours, the CMRF-56 immunoselected cells were washed, counted, and 2×10^5 cells were added in 100 μL of complete media to 8-μm transwell inserts (BD Biosciences). The plates were placed in the incubator and migration of the cells into wells containing 100 ng/mL CCL21 (R & D Systems) was assessed after 4 hours. The inserts were then removed and the content of the wells collected for counting and viability assessment by Trypan blue (Sigma). The subpopulations of migrating cells were assessed using antibody labeling and flow cytometry analysis as described earlier.

Generation of MART-1–specific CTL From CMRF-56 Immunoselected Cells

CMRF-56 immunoselected cells cultured with each of the activation stimuli for 2 or 4 hours, were washed and pulsed for 1 hour with 2 μg/mL MART-1 peptide (ELAGIGILT). After washing to remove excess peptide, CMRF-56 immunoselected cells were placed in 24-well plates at a 1:5 ratio with PBMC responders (CMRF-56 depleted cells). The cultures were supplemented with 100 ng/mL IL-7 initially and 25 U/mL IL-2 was added at day 3 and was maintained thereafter, with fresh media being added to cultures every 2 to 3 days. On day 10, the responding cells were counted and restimulated with MART-1 peptide-pulsed irradiated (3000 cGy) PBMC at a 1:10 responder ratio. On days 10 and 17, cells were labeled with MART-1 pentamer, CD8, CD3, and 7AAD to evaluate MART-1–specific CTL within the responding population by flow cytometry.

Generation of Anti-MM CTL From CMRF-56 Immunoselected Cells

MM protein lysate was prepared by 5 freeze/thaw cycles of HLA-$A2^+$ MM cell line, U266, in PBS. The solution was centrifuged at 13,000*g* to remove cell debris and the soluble protein concentration quantified using the MicroBCA Protein Assay Reagent Kit (Pierce, Rockford, IL). Protein lysate was loaded at 1 mg/mL into CMRF-56 immunoselected cells and incubated at 37°C for 2 hours,

followed by 1000 U/mL GM-CSF for a further 2 hours to activate the cells. After thorough washing, the stimulators were placed into 24-well plates with PBMC responders as described above. Responders were restimulated on days 10 and 17 using irradiated (3000 cGy) PBMC loaded overnight with MM lysate at 0.5 mg/mL at a ratio of 1 PBMC: 10 responders. The responders were analyzed by flow cytometry at days 10, 17, and 23 for T-cell and NK-cell markers, and on day 24, CTL activity was assessed by a 4-hour chromium (^{51}Cr) release assay using U266 (HLA-A2$^+$ MM), SKMEL05 (HLA-A2$^+$ melanoma), SKMEL28 (HLA-A2$^-$ melanoma), and K562 (NK-cell target) cells as targets. Specific ^{51}Cr release was quantified by Microbeta Scintillation Counter.

Statistical Analysis

All data were analyzed using analysis of variance and Bonferroni posttests were used to determine significance ($P < 0.05$). Paired student t tests were also used where indicated.

RESULTS

CMRF-56 Immunoselection Generates an Enriched Antigen-presenting Cell Population

Immunoselection of CMRF-56–positive cells from an experience of 33 separations resulted in a mean yield of 74.6% CMRF-56$^+$ DC. Further analysis of the antigen-presenting cells, namely BDC, monocytes, and B cells showed that these cells comprised 82.4 ± 4.4% of the CMRF-56–positive fraction (n = 7, Fig. 1A). BDC and monocytes predominated over B cells in the CMRF-56 selected preparations ($P < 0.05$, n = 7, Fig. 1B) but the relative proportion of B cells varied between donors. Myeloid (CD123^{lo} CD11c$^+$) rather than plasmacytoid (CD123^{hi} CD11c$^-$) BDC were, as expected,[6] the main BDC type retained in the preparations ($P < 0.001$, Fig. 1C). Of these, CD1c$^+$ myeloid BDC were the primary CD11c$^+$ subtype of BDC, comprising 20.5% ± 2.8% of the entire preparation. The CD141$^+$ and CD16$^+$ subsets of CD11c$^+$ BDC and a subset of CD11c$^+$ BDC apparently negative for these markers made up the remainder of the BDC population.

Exposure to Immune Activators Increases Costimulatory Molecule Expression on CMRF-56$^+$ BDC

When examined immediately after isolation, 67% ± 5% of the CMRF-56 immunoselected BDC expressed CD83 and 61% ± 3% expressed CD86, whereas CD40 and CD80 were expressed at much lower levels namely 2.1% ± 1.2% and 18.6% ± 6.7%, respectively (n = 4). There was no significant difference in expression of costimulatory molecules by CMRF-56 BDC after 22 hours of culture, however, after 2 hours of exposure to each of the activation stimuli, CD86 expression was significantly up-regulated ($P < 0.05$, Fig. 2). PolyI:C, CD40L, and cytokine cocktail activation also significantly increased CD83 expression ($P < 0.05$, Fig. 2). Likewise expression of CD40 and CD80 were significantly up-regulated by CD40L and cytokine cocktail activation (CD40 $P < 0.05$, CD80 $P < 0.01$, Fig. 2).

Activation of CMRF-56 Immunoselected Cells Enhances Cytokine Secretion

Cytokine production by the CMRF-56 immunoselected cells with and without activation was measured in culture supernatants. Cytokine cocktail exposure significantly increased the secretion of IL-1β by the CMRF-56 immunoselected cells, as well as the inhibitory cytokines, IL-10, and IL-6 ($P < 0.01$, Table 1). IL-8 was secreted into the culture supernatants in the absence of activation stimuli, and this increased significantly after cytokine cocktail ($P < 0.01$), CD40L and GM-CSF activation ($P < 0.05$). TNF secretion was also significantly increased with CD40L ($P < 0.01$) and cytokine cocktail activation ($P < 0.05$). Low levels of IL-12p70 that is, 3 to 6 pg/mL were secreted by CMRF-56 immunoselected cells but, interestingly, these were not increased by activation (Table 1).

Activation Improves Migration of CMRF-56 BDC Toward Chemokine CCL21

Next we explored the potential of CMRF-56 immunoselected cells to migrate toward the chemokine CCL21. Even in the absence of chemokine, spontaneous migration of BDC and B cells was observed. Significantly more BDC than monocytes migrated toward CCL21 ($P < 0.01$), and after activation, the proportion of migrating BDC exceeded that of B cells ($P < 0.05$, pooled activation data, Fig. 3A). Cytokine cocktail activation improved BDC migration, whereas GM-CSF alone had little effect. However, GM-CSF combined with 1 μg/mL PGE_2 improved BDC migration from 13.9% ± 0.5% of the loaded BDC without activation to 28% ± 3.8% with activation ($P < 0.05$, Fig. 3B).

As we have published comparative data showing that BDC generate similar peptide-specific CTL responses to monocyte-derived DC,[35] we chose to compare the migratory capacity of CMRF-56 selected BDC and monocyte-derived DC. BDC and monocyte-derived DC from the same donor were compared directly with and without GM-CSF and PGE_2 or cytokine cocktail activation. Activated BDC had a significantly enhanced migratory capacity compared with monocyte-derived DC in 3 of the 4 patients tested (Fig. 3C).

Induction of MART-1–specific CTL by Activated CMRF-56 Immunoselected Cells

Peptide-pulsed CMRF-56 preparations activated with the defined stimuli were assessed for the ability to induce MART-1–specific CTL as determined by pentamer staining. The activated CMRF-56 immunoselected cells all generated increased CTL responses, when compared with the untreated control. The TLR ligands polyI:C and imiquimod, and the cytokine cocktail required an exposure time of 4 rather than 2 hours to induce specific responses (Fig. 4). GM-CSF activation for

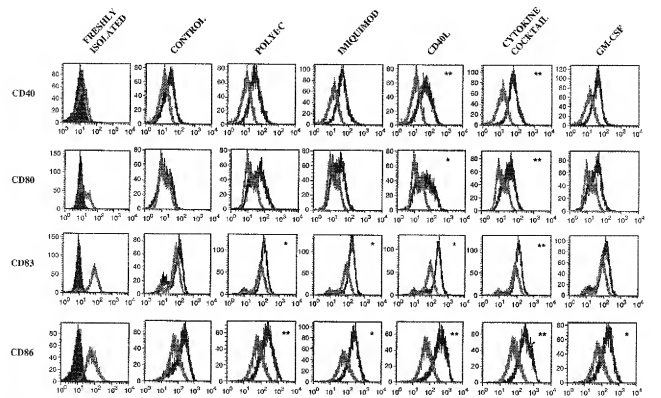

FIGURE 2. Phenotype of CMRF-56 immunoselected BDC before and after exposure to activation stimuli. CMRF-56 immunoselected cells were analyzed by flow cytometry to determine costimulatory molecule expression on BDC directly after isolation (freshly isolated) or after 24 hours of culture including an initial 2-hour exposure to the activation stimulus indicated. The isotype control (filled histogram) is shown in the first column only for clarity. The freshly isolated phenotype and phenotype after activation and culture are represented by the gray histogram and the black histogram, respectively. Significant up-regulation of costimulatory molecules from the initial postisolation phenotype is indicated by $P<0.05$ (*) and $P<0.01$ (**).

2 hours resulted in significantly better MART-1–specific CTL responses compared with CMRF-56 immunoselected cells without activation ($P = 0.03$, n = 5, Fig. 4), whereas a 4-hour activation period seemed less effective ($P = 0.46$, n = 5, Fig. 4).

The Effect of Activation on the Generation of U266-specific CTL

The HLA-A2$^+$ MM cell line U266 protein lysate was loaded into CMRF-56 immunoselected cells and used to stimulate PBMC. CD8 and CD4T cells were expanded in similar proportions by lysate loaded CMRF-56 immunoselected cells irrespective of the activation stimulus used (24.57% ± 1.9% CD8T cells, 55.81% ± 5.7% CD4T cells, n = 3). However, CTL primed by GM-CSF activated CMRF-56 immunoselected cells lysed U266 cells more efficiently than those primed with cells that were not activated (GM-CSF 40.9% ± 7.3% vs. no activation 18.8% ± 4.3%, effector:target ratio 25:1, $P = 0.051$, n = 3, Fig. 5), or those that were activated

TABLE 1. Cytokine Secretion by CMRF-56 Immunoselected Cells After Exposure to Activation Stimuli

	T_H1 Inducing Cytokines		T_H2 Inducing Cytokines		Proinflammatory Cytokines	
	IL-12p70	TNF	IL-6	IL-10	IL-1β	IL-8
No activation	3.1 ± 1.4	4.3 ± 1.0	8.9 ± 5.8	1.9 ± 1.1	5.1 ± 3.1	631.3 ± 324.1
PolyI:C	5.7 ± 1.0	14.8 ± 4.6	14.7 ± 5.9	4.7 ± 0.8	2.5 ± 2.5	348.0 ± 40.9
Imiquimod	6.0 ± 2.4	14.3 ± 4.4	8.9 ± 1.6	7.2 ± 1.3	8.0 ± 2.7	1051.7 ± 487.5
CD40L	4.6 ± 1.9	45.2 ± 11.3**	9.2 ± 2.2	6.3 ± 1.0	8.1 ± 5.0	3065.6 ± 882.1*
Cytokine Cocktail	4.4 ± 2.1	35.4 ± 2.6*	5459.8 ± 764.8**	28.6 ± 10.5**	119.3 ± 8.9**	11268.4 ± 836**
GM-CSF	4.9 ± 2.2	18.6 ± 5.9	6.6 ± 1.0	4.9 ± 1.1	9.0 ± 5.2	2367.7 ± 402.4*

After immunoselection, CMRF-56 cells were activated for a period of 2 h, washed and replaced into culture for a further 22 h. Supernatants were collected and analyzed using a cytokine bead array kit. Values are in pg/mL. *$P < 0.05$; **$P < 0.01$.

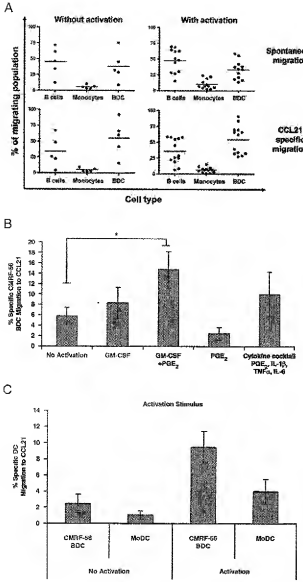

FIGURE 3. Migration of cell subsets within the CMRF-56 preparation. After isolation, CMRF-56 immunoselected cells were activated for 2 hours, and allowed to migrate for 4 hours in the presence or absence of chemokine CCL21. The cells that migrated were analyzed by flow cytometry (n = 5). A, CMRF-56 immunoselected B cells and BDC migrated in the absence of chemokine stimulation but BDC migrated more specifically in the presence of CCL21. The proportion of migrating cells was unchanged after activation with all the stimuli. B, Migration of activated CMRF-56 BDC in the presence of CCL21. GM-CSF and PGE_2 induced significantly greater migration (*P<0.05). C, CCL21-specific migration of CMRF-56 BDC and MoDC generated from the same donors. CMRF-56 cells were activated with GM-CSF combined with PGE_2 and MoDC were activated with the cytokine cocktail. The mean and standard errors of the overall results from 4 different donors are indicated. In 3 of the 4 cases, BDC had superior migration.

with CD40L (Fig. 5). Killing of U266 cells by CTL primed with GM-CSF activated CMRF-56 cells was significantly higher than that of the HLA-$A2^+$ matched melanoma cell line SKMEL05 and HLA-$A2^-$ mismatched melanoma cell line SKMEL28 (25:1, $P < 0.05$, n = 3) and higher than NK cell K562 killing (25:1, $P = 0.07$, n = 3).

DISCUSSION

We reported previously that circulating BDC can be harvested in clinically relevant numbers after leukapheresis[7,8] and that, when cultured as part of the PBMC population, BDC survive longer and mature in vitro, without the addition of exogenous cytokines.[6] Having shown that BDC can be positively immunoselected using CMRF-56 mAb,[7] we undertook this study to characterize this novel BDC preparation in more detail: notably, its cellular constitution, response to clinically appropriate activators, ability to cross-present tumor antigens, and generate tumor-specific CTL responses. After examining a panel of clinically applicable DC activation stimuli, we identified GM-CSF as effective not only in improving peptide and protein CTL responses by CMRF-56 immunoselected cells, but also in increasing BDC migration when combined with PGE_2. Indeed the GM-CSF/PGE_2 activated CMRF-56 selected cells had significantly greater migratory capacity than cytokine cocktail activated MoDC. These new data increase the case to test CMRF-56 immunoselected cells in clinical therapeutic vaccination trials.

The simple 1-step CMRF-56–positive immunoselection method yields a cell preparation enriched in antigen-presenting cells, predominantly, BDC and monocytes. The $CD1c^+$ myeloid BDC subset accounts for the majority of the BDC present, but smaller populations of $CD141^+$ and $CD16^+$ BDC were also identified. A further myeloid BDC population lacking all 3 BDC subset markers was also identified but these are likely to belong to the $CD16^+$ subset, as labeling with biotinylated CMRF-56 mAb has been observed to block CD16 binding (data not shown), most probably through the Fc region.[33] The CMRF-56 preparation contained an average of 17.6% ± 4.4% unwanted cells including T cells, NK cells, natural killer T cells, and HLA-DR–negative cells, that may have coseparated with antigen-presenting cells as a result of cellular aggregates formed during overnight incubation.[34]

Short activation periods of 2 and 4 hours were chosen to initiate BDC maturation to optimize BDC viability as BDC, including CMRF-56 BDC, do not survive well in vitro without cytokine support.[6,32,35] Although time frames of 24 to 48 hours are generally used to induce MoDC maturation, transient activation has also been shown to induce mature, migratory DC in vitro[27,36] and in vivo.[27] There is also evidence that prolonged activation induces IL-12p70 secreting, proinflammatory type DC but these cells do not migrate, suggesting that transiently activated, migratory BDC may

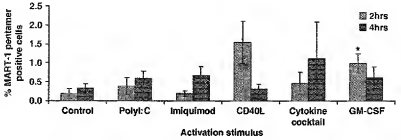

FIGURE 4. Induction of MART-1 peptide-specific CTL by activated CMRF-56 cells. After isolation and activation of CMRF-56 cells for 2 or 4 hours, cells were pulsed with MART-1 HLA-A*201 restricted peptide (ELAGIGILT) and added to CMRF-56–negative PBMC responders at 1:5 ratio. Cultures were restimulated using 1:10 peptide-pulsed PBMC on day 10. At day 17 the percentage of MART-1–specific CD8T cells in each culture was assessed by pentamer analysis. Data were analyzed using student *t* tests comparing activation to control wells. *$P<0.05$.

be better for tumor immunotherapy, despite producing barely detectable levels of IL-12p70.[12,29] Furthermore, transiently activated DC producing low levels of IL-12p70 apparently respond with increased IL-12p70 production after a second stimulus.[19] This would allow DC, which migrate after injection to become proinflammatory upon contact with responding T cells, an ideal outcome after in vivo administration.[36]

After isolation, BDC within the CMRF-56 preparation displayed an intermediate phenotype, which developed, after exposure to activation stimuli, into a mature BDC phenotype, particularly in the presence of CD40L and cytokine cocktail. The leukocyte recruiting cytokine IL-8 was the only cytokine produced in high levels by CMRF-56 cells without activation. Activation of the CMRF-56 preparation with cytokine cocktail resulted in proinflammatory type DC, with much higher secretion of most cytokines measured, whereas GM-CSF and CD40L activation seemed to slightly increase the T_H1 cytokine TNF, but not the T_H2 cytokines IL-6 and IL-10. These results suggest, that although CMRF-56 immunoselected preparations contain significant number of monocytes and B cells, they do not compromise the BDC, which undergo a predictable physiologic differentiation and activation process, similar to that documented using purified $CD11c^+$ and $CD1c^+$ BDC.[13]

The ability of the CMRF-56 immunoselected preparation to induce effective CTL responses after exposure to various potential clinical activators was also tested as it was possible that the presence of the monocytes, B cells, and other unwanted cells would modify the responses. The CMRF-56 cells were able to induce strong CD4T cell responses to recall antigen tetanus toxoid protein as previously described,[32] however, as observed with $CD1c^+$ BDC,[28] activation made no difference to this response (data not shown). Strong CTL responses were induced by CMRF-56 cells pulsed with a peptide derived from influenza matrix protein as previously observed,[7,32] however, activation also failed to improve this recall response (data not shown). We then examined the influence of CMRF-56 cell activation on the generation of primary CTL responses as this is considered crucial for an effective antitumor response.[16,37] Activation of CMRF-56 cells, especially by GM-CSF, improved the induction of specific primary CTL responses to the tumor-associated antigen, MART-1 peptide. Exposure to certain activation stimuli for 2 hours was sufficient and optimal for efficient CTL priming.

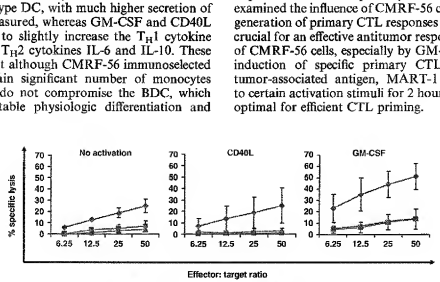

FIGURE 5. The effect of CMRF-56 cell activation on the induction of anti-MM CTL activity. CMRF-56 cells loaded with myeloma cell lysate were activated with CD40L or GM-CSF and added in a 1:5 ratio to responding CMRF-56–negative PBMC. After 2 restimulations on days 10 and 17 using 1:10 lysate-loaded irradiated PBMC, chromium release assays were performed using U266 ($HLA\text{-}A2^+$), SKMEL05 ($HLA\text{-}A2^+$), and SKMEL28 ($HLA\text{-}A2^-$) targets. Comparison of CTL activity on targets with primed with CMRF-56 cells with no activation, CD40L activation or GM-CSF activation. #$P=0.051$.

Interestingly, the MART-1 peptide-specific responses were induced, despite the extremely low levels of IL-12p70 secretion by CMRF-56$^+$ cells. Many reports suggest that BDC are not major produces of IL-12p70 and that they may have IL-12 independent means of inducing CTL activity.[10,12,26,29,32,38,39] MoDC have also been reported to induce T_H1 responses in the absence of IL-12p70[31] but most investigators have assumed IL-12 production by MoDC is crucial for CTL induction. It was also notable that CD40L and cytokine cocktail, which induced the highest up-regulation of BDC costimulatory molecules did not consistently improve CTL priming with MART-1 peptide, suggesting that high costimulatory molecule expression likewise does not necessarily indicate the ability of BDC to prime CTL responses.

As the ability of CMRF-56 selected BDC to process and cross-present intact antigen to CTL had not been tested before, we used myeloma cell lysate as a test antigen system. CMRF-56 selected cells loaded with myeloma antigens induced strong specific CTL activity and this was further enhanced after activation with GM-CSF but not CD40L. This indicates that GM-CSF activation not only permits but also enhances cross-presentation of antigens. Curiously, CD40L activation did not, consistent with data obtained by others using CD1c$^+$ BDC preparations[28] and this may predict for clinical outcomes. GM-CSF is not generally considered a DC activation stimulus per se, however, it is used widely for the generation and maintenance of DC in vitro and has recently been shown to polarize CD1c$^+$ BDC to generate T_H1 responses.[29] In vivo mouse data indicates that GM-CSF is essential for CTL generation by DC exposed to soluble antigen[40] supporting the view that GM-CSF may facilitate cross-presentation. Thus, GM-CSF becomes a logical candidate for BDC activation in future clinical trials.

CMRF-56 immunoselected BDC were capable of specific migration to the secondary lymphoid organ chemokine CCL21, without additional activation, in a manner reminiscent of CD1c$^+$ BDC.[12] GM-CSF activation alone did not improve their migration, or did activation with the cytokine cocktail containing PGE_2 or PGE_2 alone. PGE_2 has been identified as essential for MoDC migration and shown to aid CD1c$^+$ BDC migration in vitro[10,12,41,42] and Langerhans cells in vivo,[43] although it has no effect on the CCL21 receptor, CCR7 levels. The addition of PGE_2 to GM-CSF during the 2-hour activation phase, increased CCL21-specific BDC migration significantly. Given the relatively poor migration of MoDC in vivo[18] and this data showing that suitably activated BDC migrate more effectively than MoDC in vitro, it will be important to document the migratory capacity of CMRF-56 BDC in any future clinical trial.

Another type of BDC preparation, APC8015, is currently being used in clinical trials for the treatment of prostate cancer patients. This preparation contains on average 18.6% BDC, 65.1% T cells, 16.6% monocytes, and 5.0% B cells and is exposed to GM-CSF conjugated to the tumor-associated antigen protein prostate acid phosphatase.[44] In comparison to the CMRF-56 immunoselected preparation, APC8015 contains substantially fewer BDC and other antigen-presenting cells. Nonetheless, APC8015 has induced cellular immune responses in prostate cancer patients, proving that BDC vaccination can induce effective antitumor responses in vivo.[15,45]

We showed previously that BDC could be obtained from MM patients using CMRF-56 immunoselection and that these preparations generated control peptide-specific CTL responses.[32] These new data further define the characteristics and functional capacities of the CMRF-56 immunoselected cells. In particular, it defines how these cells may be activated to improve CTL generation, while maintaining their migratory capacity. Critically, it also shows that the CMRF-56 immunoselected BDC preparations can cross-present protein antigen to generate strong antimyeloma CTL responses. These data provide the essential preclinical information to justify the use of CMRF-56 immunoselected BDC preparations in a phase 1 clinical study to assess their safety and ability to generate MM-specific immune responses in MM patients with minimal residual disease. Such a study should include the capacity to test the resulting CTL responses against primary MM samples.

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